Functional Transport of Organic Anions and Cations in the Murine Mesonephros

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Abstract

The mesonephros of mammals is a transient renal structure that contributes to various aspects of mammalian fetal development including the male reproductive system, hematopoietic stem cells and vascular endothelial cells. The mesonephros develops from the intermediate mesoderm and forms tubules that are segmented in a similar way to the nephrons of the permanent kidney (but lacking loops of Henle). Early studies have suggested that the mesonephros in marsupials and some placental mammals may perform an excretory function, but these studies have not directly shown active transport of organic anions and cations. Excretory function in the rodent mesonephros has not been investigated. Functional characterisation of the earliest stages of mammalian renal development is important for our understanding of congenital disease and may help to inform the growing field of renal tissue engineering. Here, we use live uptake and efflux assays *in vitro* to show that the murine mesonephros is able to transport organic anions and cations through specific transporters from early in its development. Transcript analysis suggests that there are subtle differences between the transporters involved in uptake and efflux by the murine permanent, metanephric tubules and by the mesonephric tubules. These data suggest that the mammalian mesonephros can provide an excretory function for the early developing embryo, in addition to the excretory function provided by the placenta.

Introduction

In lower vertebrates the mesonephros is the final and definitive renal structure, performing an excretory function for the organism. In mammals, the mesonephros is a temporary structure that is eventually replaced by the metanephros, the permanent kidney. The mammalian mesonephros develops parallel to the nephric duct (formed from condensed intermediate mesoderm) as the duct elongates along either side of the midline in a cranial to caudal direction.(21) This primitive kidney consists of segmented tubules that connect to the adjacent nephric duct, making an arcade of tubules draining into a common duct that initially drains into the cloaca and, later, into the urogenital sinus. In mice, the mesonephros begins to develop from around embryonic day 9.5 (E9.5), with tubules continuing to form caudally alongside the nephric duct. The most cranial 4-6 tubules of the mesonephros are attached to the nephric duct and a further 14-20 will remain unattached whereas, in humans, all the mesonephric tubules will connect to the nephric duct. As the permanent kidney develops, the mammalian mesonephros regresses and will disappear by E14 in mouse, with some tubules being modified in males to contribute to the male reproductive system.

The degree to which the mammalian mesonephros is functional, during its brief existence, remains unclear. Previous studies in rabbits and pigs have shown that their mesonephroi may be capable of producing urine, although attempts to demonstrate filtration by analysing the composition of the allantoic fluid(3, 4, 14) or bladder contents(24) were inconclusive. One very early study in the opossum (a marsupial in which the mesonephros persists for at least 10 days after birth), involving injection of ferrocyanide and phenol red followed by investigation of mesonephric fluids, suggested that some glomerular and tubular transport may have occurred.(6) In addition, *in vitro* microperfusion procedures suggest that volume transport rates of tritiated water per unit area are similar between mesonephric tubules and metanephric proximal tubules in rabbits(27), and transepithelial ion transport in perfused *in vitro* rabbit mesonephric tubules has been demonstrated by examining transmembrane electrical cell potential difference(26). However, these studies have not directly shown active transport; in particular, the uptake and efflux of organic anions and cations in the rodent mesonephros has not been studied.

The nephron is the functional unit of the metanephric (permanent) kidney. Transepithelial transport across nephron tubules is an important mechanism for reabsorption and excretion of endogenous and xenobiotic compounds. Anions in the mouse metanephric kidney are transported into the tubular cells by the organic anion transporters (OATs) of the SLC22A family, including the basolateral Oat1 and Oat3, the apical Oat2, Oat5 and Urat1, as well as some members of the selective SLCO family(1, 8, 19). Hydrophilic anion efflux into the lumen of the tubules is primarily mediated by the apically-located MRP (ABCC) family of efflux proteins and to some extent the efflux transporter Breast Cancer Resistance Protein (BCRP).(17) Another important extrusion protein at the apical brush border is P-glycoprotein (MDR1) but this efflux pump mainly mediates extrusion of hydrophobic or neutral compounds.(28) In addition to these anion transporters, uptake of organic cations in mouse metanephric kidneys is mediated mainly by Oct1 and Oct2 (also part of the SLC22A family). The H+ antiporter Mate1 is another cation transporter in the murine metanephros, acting as an extrusion or uptake transporter depending on the concentration of H+ in the intracellular compartment relative to the external environment.(25)

When used with specific inhibitors of individual transporters or families of transporters, fluorescent anions or cations such as 6-carboxyfluorescein (6-CF, 6FAM) and DAPI can be employed as tracers to examine transporter activity in live cultures *in vitro*. Due to morphological similarities between mesonephric and metanephric tubules, we hypothesised that murine mesonephric kidney tubules would also be capable of transepithelial transport through apical and basolateral uptake and efflux proteins.

Here, we show that organic anion and cation transporters are expressed in the murine mesonephros at least from E10.5. We also show expression of two efflux pumps in the murine mesonephros that are known to be important extrusion proteins in the tubules of the metanephros. Using a combination of published uptake assays and an efflux assay,(12) we show that the murine mesonephros is capable of inhibitable uptake and efflux of organic anions, and uptake of cations in culture. These data add to previous work showing accumulation of fluid from the

mesonephros in other mammalian species, and suggest that the mammalian mesonephric kidney functions in a similar way to the metanephric kidney, providing an early excretory system for the mammalian embryo and acting in parallel with the placenta.

Materials and Methods

Dissection and culture of mouse mesonephroi

Embryos from timed mated CD1 females were removed from the uterus and the embryonic mesonephroi were dissected using standard techniques.(16) Gonads were carefully removed, leaving as many mesonephric tubules as possible intact. Mesonephroi were placed in culture dishes using the Sebinger method, for optimal imaging,(22) and then assayed directly in the dishes.

Chemicals and Reagents

Metformin hydrochloride (PHR1084) and cimetidine (C4522) were purchased from Sigma Aldrich. 6-carboxyfluorescein (C1360) was purchased from Invitrogen. Probenecid (P36400) was purchased from Life Technologies. Probenecid, metformin, cimetidine and 6-carboxyfluorescein were dissolved in water to make stock concentrations as follows: probenecid 250mM, metformin 100mM, cimetidine 25mM, 6-carboxyfluorescein 1mM. Dilutions for live assays were diluted in kidney culture medium: see below.

Transport assays

Assays were carried out on live cultures in the Sebinger culture system using kidney culture medium (KCM: Eagle's MEM with Earle's salts [Sigma, M5650)],10% FCS [Invitrogen, 10108165] and 1% Penicillin/Streptomycin stock [Sigma, P4333]). For imaging purposes, all uptake and efflux assays were carried out in the presence of rhodamine-conjugated peanut agglutinin (20μg/ml, Vector Laboratories, RL-1072) which highlights morphological structures. Anion uptake assays were performed by treating the mesonephroi in Sebinger culture dishes for 1 hour at 37ºC with the fluorescent anion 6-carboxyfluorecein (1 μM) either with probenecid (2.5 mM in KCM) or with only culture medium. Cultures were washed once in PBS and then

incubated with probenecid (10mM) for 15 min at 37ºC to trap any intracellular fluorophore before imaging. Efflux of anions was assayed by pre-loading all the cultured mesonephroi with 6-carboxyfluorescein (1 μM) in KCM for 1 hour at 37ºC, washing once in PBS, imaging briefly to confirm uptake, then incubating with either probenecid to block anion efflux (10mM, in KCM) or plain KCM culture medium for 30-60 minutes at 37ºC. Cultures were washed in PBS before imaging. Uptake of cations by the tubules of the mesonephroi was assayed by incubating live cultures with the fluorescent cation 4′,6-Diamidino-2- phenylindole (DAPI) (1 μM in KCM) for 30 minutes either alone or in the presence of the cation transporter inhibitors cimetidine (200 μM) and metformin (5mM). DAPI is bound stably to DNA once inside the cell nucleus and therefore the cultured mesonephroi could be imaged directly without the need to block efflux.

Annexin V staining

An Annexin V assay kit was used (BioVision; K103) for staining apopototic nuclei. E11.5 mesonephroi were placed in Sebinger culture and incubated with DAPI without inhibitor as described above. The cultures were washed 3 times for 10 min at room temperature before incubating with Annexin V binding buffer for 10 min followed by incubation with Annexin V 1:100 in binding buffer for 5 min at 37ºC. Cultures were kept in the dark and imaged immediately, directly in the culture dish, using a Nikon A1R inverted microscope (using TIRF attachment). Images were analysed with FIJI data analysis software.

RT-PCR analysis

Mesonephroi were dissected from the embryos of timed mated CD1 females at embryonic day E10.5 (20 pooled mesonephroi), E11.5 (20 pooled mesonephroi) or E12.5 (15 pooled mesonphroi) taking care to discard the gonads during dissection. Metanephroi were dissected from embryos of timed mated CD1 females at E13.5 (15 pooled metanephroi) or E15.5 (10 pooled metnephroi). Adult mouse kidney RNA was extracted from a single adult male CD1 mouse. RNA was extracted using the RNeasy mini kit (Qiagen, Cat no. 74104) and RNA concentrations were determined using the Nanodrop system. cDNA was made using 2 μg of RNA. Purity of RNA and absence of genomic DNA was confirmed by PCR for β-actin using primers that

span an intron (Table 1) and using cDNA preparations made with or without addition of reverse transcriptase. PCRs for genes of interest were carried out in multiplex reactions with primers for β-actin acting as loading controls. Less cDNA was used in the positive control reactions in the gene-specific PCRs (from adult male) due to relatively large amount of transporter transcript in adult kidneys (0.5 μl instead of 2 μl of cDNA, equivalent to 33 ng and 133 ng of RNA respectively).

Table 1

Primers for RT-PCR

Oat1
Forward primer: ATGGTGGGAGTGTTACTGGG
Reverse primer: GGAGCCGGAAAATGCAGTAG
Oat3
Forward primer: TACAGTTGTCCGTGTCTGC
Reverse primer: TTCAGCTCCTCCACAGTGAG
Oct1
Forward primer: GTCCTTCGTTTGCAGACCTG
Reverse primer: TATTGGGTAGATGCGGCCA
Oct2
Forward primer: TGGGCATTGGTTACCTAGCA
Reverse primer: TTGCTGACCAGTCCCTGTAG
Mate1
Forward primer: ACTACCTGTCAGACCACGTG
Reverse primer: GGACGGATAGGCAAAGCTTG
Mrp2
Forward primer: ACACCAACCAGAAATGCGTC
Reverse primer: GGACAGAACAAAGCCCACAG
Bcrp
Forward primer: GAGTGGGTTTCTAGTCCGGA
Reverse primer: GAAATGGGCAGGTTGAGGTG
β-actin
Forward primer: CTGGGACGACATGGAGGARA*
Reverser primer: AAGGAAGGCTGGAARAGWGC*

- R=50:50 A+G, W=50:50 A+T

Animals

This project involved no experiments conducted in living animals. Embryonic tissues used for post-mortem culture were obtained from healthy CD1 mice killed by trained staff of the UK Home Office-licenced animal house under Schedule 1 of the UK Animals (Scientific Procedures) Act 1986.

Results

Uptake and efflux of fluorescent anions in the murine mesonephros and expression of organic anion transporters

We have previously shown that murine embryonic kidneys developing *in vitro* are able to transport organic anions and cations using a series of uptake and efflux assays, and that they express known transporters both *in vivo* and *in vitro.(12)* Using a similar approach, we investigated the ability of the murine mesonephric tubules to take up the fluorescent anion 6-carboxyfluorescein (6-CF, 6-FAM). Transport activity of organic anion uptake transporters can be competitively inhibited by the uricosuric drug probenecid.(5, 15, 30) In this assay, the mesonephros is isolated from the murine embryo, and is then treated *in vitro* with the fluorescent anion 6-CF either with or without the inhibitor probenecid. Any accumulated fluorophore is then trapped in the cells that have taken it up by treating all samples with a high concentration of probenecid (Fig 1A). Mesonephric tubules took up 6-CF *in vitro* at E10.5, E11.5 and E12.5, and this uptake was abolished by the presence of probenecid, indicating specific uptake function in the mesonephric tubules through organic anion transporters (Fig 1B). Uptake assays were repeated 4 times (E10.5), 7 times (E11.5) and 2 times (E12.5) with all cultures assayed for each age displaying appropriate uptake (without probenecid) or blockage of uptake (treated with probenecid). Transcript analysis by semi-quantitative multiplex RT-PCR indicated that Oat1 and Oat3 transcripts were detectable from E10.5 to E12.5 (Fig 1C).

Transepithelial transport is an important mechanism for extrusion of organic ions and xenobiotics by renal tubules. Having shown that mouse mesonephric tubules can take up fluorescent anions through specific transporters, we next investigated their ability to efflux fluorescent anions. In the uptake assay, intracellular accumulation of the fluorescent anion 6-CF is inhibited by probenecid. In the retention assay, cells

are allowed to take up the fluorescent anion and then washed, followed by treatment with probenecid to block efflux, or with vehicle only to allow efflux through known extrusion pumps. Retention of the fluorophore when treated with probenecid indicates specific inhibition of the efflux transporters (Fig 2A). Mesonephric tubules were able to efflux 6-CF *in vitro* over the course of 15 minutes at E10.5, E11.5 and E12.5, and retention of the fluorophore in cultures treated with probenecid indicated that this transport could be inhibited specifically (Fig 2B). Anion efflux assays were repeated 3 times (E10.5), 4 times (E11.5), and 2 times (E12.5), with all cultures assayed at each age displaying appropriate efflux (without probenecid) or fluorophore retention (treated with probenecid). Transcript analysis of Mrp2 (an important anion efflux protein expressed in the proximal tubules of the developing murine metanephros)(10) and Bcrp (an efflux pump that can transport some anions) by multiplex RT-PCR indicated that Mrp2 is expressed from E10.5 to E12.5 with peak expression at E11.5, and that Bcrp is expressed in the mesonephric tubules of all ages analysed.

Uptake of fluorescent cations in the murine mesonephros and expression of organic cation transporters

Next, we qualitatively investigated the uptake of cations in the murine mesonephros by investigating the uptake of the cationic molecule 4′,6-Diamidino-2-phenylindole (DAPI) into the tubules through cation transporters, and probing the specificity of uptake using competitive inhibitors of the Oct and Mate families, namely, cimetidine and metformin(2, 7, 11, 18, 23) (Fig 3A). The cationic compound DAPI does not easily cross plasma membranes except via specific cation transporters, and emits blue fluorescence with higher intensity once intercalated with double-stranded DNA. Accumulation of blue fluorescence in the cells can therefore be taken to indicate transport across the epithelial cell membrane through cation transporters. Uptake of DAPI was observed in murine mesonephroi *in vitro* at E10.5, E11.5 and E12.5, and this accumulation was reduced or abolished when the cultures were co-treated with cimetidine and metformin (Fig 3B). Each experiment included both control (no inhibitor) and treated (with inhibitor) mesonephroi. For E10.5 mesonephroi, appropriate uptake of DAPI (control; without cimetidine and metformin) or no uptake of DAPI (treated; with cimetidine and metformin) was observed in 5/6 experiments. For E11.5, appropriate uptake of DAPI (control) or no uptake (treated)

was observed in 2/3 experiments. For E12.5, appropriate uptake of DAPI (control) or no uptake (treated) was seen in every experiment. In 1/6 experiments (E10.5) and 1/3 experiments (E11.5) no uptake of DAPI was seen in either control or treated cultures. This is likely due to a technical issue with the Sebinger culture system that is prone to drying, but we cannot exclude the possibility that in a small proportion of mesonephroi of these ages, the cation transporters are not being expressed. In cultured mesonephroi some diffuse fluorescence was seen in all cultures regardless of treatment. This had previously been observed in metanephric cultures and was shown to be due to DAPI binding to the nuclei of apoptotic or dead cells with disrupted membranes, since these spots co-localised with the apoptotic marker Annexin V.(12) We tested this in our mesonpehric cultures (Figure 3D), performing the cation uptake assay (without inhibitors) and then incubating with Annexin V. No Annexin V staining was seen in the DAPI-positive tubules, and Annexin V was seen to co-localise with bright, dense DAPI-positive nuclei outside the tubules indicating that the diffuse DAPI staining seen in these cultures are dead or dying cells. Using semi-quantitative multiplex RT-PCR we investigated the expression of known cation transporters in the murine mesonephros. We found that Oct1 and Oct2 transcripts were not detected, whereas Mate1 transcript was detected at all ages analysed (Fig 3C).

Discussion

The mammalian mesonephros is thought to confer some excretory function mainly due to the presence of glomeruli and likely production of urine, with previous studies focussed on larger mammals due to their larger and more well-developed mesonephroi. Directional transport across the tubule epithelia has been suggested by earlier studies, but the functionality of the mesonephros in rodents has not been studied. The mesonephros contributes to other aspects of mammalian development including the development of the male reproductive system, hematopoietic stem cells and vascular endothelial cells,(20) giving evolutionary rationale for the retention of this transient structure during mammalian development. Excretion and reabsorption of waste products and endogenous compounds by the mesonephros in the early mammalian embryo may also be important for development.

In this study, the tubules of the murine mesonephros was shown to be capable of both uptake and efflux of a fluorescent organic anion through membrane transporters

in an inhibitable manner. Transcripts for organic anion uptake transporters were detected in all ages investigated. In addition, the cationic compound DAPI was taken up by the tubules and this uptake could be competitively inhibited by co-treatment with inhibitors of the Oct and Mate families, cimetidine and metformin. DAPI has been shown in cultured human kidney cells to be a substrate for the renal organic cation transporters MATE1, MATE2K and OCT1, but not OCT2, OCT3 or the OCTN family.(29) As with humans, mice express Mate1 and Oct2 in the metanephric kidneys, and in contrast to humans, mice also express Oct1 in the kidneys (in humans, Oct1 is expressed mainly the liver).(9) Murine Mate2 is not expressed in the kidney, with expression highest in the testis in males and the colon in females(13) underlining species differences. Of the possible murine renal cation transporters for which DAPI is a substrate, only Mate1 transcript was detected, indicating a different expression pattern than in the murine metanephros, where Oct1 and Oct2 are also expressed. The absence of Oct1 transcript suggests that uptake of DAPI in the murine mesonephric tubules is through the cation transporter Mate1. Mate1 is considered to be an extrusion protein for cations physiologically, with many compounds transported by Mate1 being exchanged for luminal H+, driving extrusion of cations into the lumen at the apical surface. However, DAPI has been shown to be taken up by Mate1 in a manner independent of H+ concentration or pH,(29) suggesting that at least some cations can be transported by Mate1 in a facilitative manner. In addition, acid-base regulation in fetal development is largely carried out by maternal mechanisms, and therefore the luminal H+ gradient seen in the post-natal kidney is not likely to be present in the mesonephros. Access to Mate1 by DAPI at the luminal surface is possible in our culture system because the Wolffian duct is severed during dissection, leaving the tubular system open at one end. Another possibility is that Mate1 is basolaterally located in the rodent mesonephros in contrast to the metanephros. There may also be an unknown cation transporter not yet identified that mediates cation uptake at the basolateral surface of the E10.5 to E12.5 rodent mesonephros.

This study adds to earlier work that suggests the mesonephros in some mammals is capable of excretory function. The presence of transcripts for known uptake and efflux transporters suggests that transepithelial reabsorption and excretion of xenobiotics and endogenous compounds occurs through known basolateral and apically localised transport proteins in addition to glomerular filtration. Together these

data offer evidence that the rodent mesonephros provides an early excretory mechanism for the developing embryo as well as its contribution to later developmental structures.

Acknowledgements and author contributions

M.L. Conception and design, acquisition and interpretation of data, drafting and revising the manuscript and figures. J.S. acquisition of data. J.D. Conception and design, interpretation of data, manuscript editing. All authors reviewed the manuscript. We thank Chris Mills for reviewing the manuscript. This work was supported by the Medical Research Council, grant MR/K010735/1 and by Kidney Research UK, grant RP_002_20160223.

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Figure legends

Figure 1

Murine mesonephric tubules transport organic anions. (A) Anion uptake assay using specific anion transport inhibitor probenecid. (B) Specific tubular uptake of the anion 6-CF in tubules of E10.5, E11.5 and E12.5 murine mesonephroi. Scale bars represent 300 μm. (C) RT-PCR analysis of basolateral anion uptake transporters. Meta refers to the embryonic metanephros at the embryonic date specified. PC is positive control (adult male kidney) and NC is negative control (no template).

Figure 2

Efflux of anions in murine mesonephric tubules. (A) Anion efflux assay (retention assay) using the specific anion transport inhibitor probenecid. (B) Specific tubular efflux of the anion 6-CF in tubules of E10.5, E11.5 and E12.5 murine mesonephroi. Scale bars represent 200 μm. (C) RT-PCR analysis of apical anion efflux transporters. Meta refers to the embryonic metanephros at the embryonic date specified. PC is positive control (adult male kidney) and NC is negative control (no template).

Figure 3

Murine mesonephric tubules transport organic cations. (A) Cation uptake assay using specific cation transporter inhibitors metformin and cimetidine. (B) Specific tubular efflux of the cation DAPI in tubules of E10.5, E11.5 and E12.5 murine mesonephroi. Yellow arrows indicate tubules. Scale bars represent 300 μm. (C) RT-PCR analysis of basolateral and apical cation transporters. Meta refers to the embryonic metanephros at the embryonic date specified. PC is positive control (adult male kidney) and NC is negative control (no template). (D) Uptake of DAPI in E11.5 mesonephros in vitro, followed by incubation with Cy-5-conjugated Annexin V (which stains apoptotic cells). Annexin V colocalises with apoptotic DAPI-positive nuclei outside the tubules (box 1, box 3) but not with DAPI-positive cells within the tubules (box 2). Scale bars represent 50 μm.